

NOVEL SYNTHETIC ANALOGUES OF IL-10 REGULATE THE BINDING OF NF-kappaB COMPLEXES TO p53 and IL-8 kappaB MOTIFS

Christian Grønhøj Larsen, Claus Johansen, Lars Iversen, Arne Holm, Borbala Gesser
Department of Dermatology, Marselisborg Hospital, University of Aarhus, DK-8000 Aarhus C, Denmark.
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INTRODUCTION

NF-kB is a transcription factor involved in the regulation of inflammation and growth in both benign and malignant cells (Gilmore et al., 1996). We have recently shown that in hepatocarcinoma cells both IL-10 and IT9302 (-Ala-Tyr-Met-Thr-Met-Lys-Ile-Arg-Asn), a nona-peptide homologous to IL-10's COOH terminal domain, is regulating NF-kB complexes by inducing NF-kB 50 and IxB- α and thereby inducing apoptosis (Gesser et al., 2002). In this study we tested whether IL-10, IT9302 and two novel IL-10 analogues are able to regulate NF-kB binding to specific "kB motifs" in monocytes as well as in a monocytic cell line U937. We constructed novel IL-10 analogues by modifying the amino-acid composition of IT9302, substituting single amino acids with natural or non-natural amino-acids.

IL-10 is known for inhibiting Nuclear Factor kB activation (Wang et al., 1995) and for stabilizing Inhibitory kB- α in monocytes (Shames et al., (1998). IL-10 is also blocking kB kinase activation and induced NF-kB p50/p50 in the monocytic cell line U937 (Schonelius et al., 1999). Specific "kB-motifs" are identified for IL-8-Mukaida et al., (1990), Harant et al., (1998) as well as for p53, Kirch et al., (1999).

We find that both IL-10 and IT9302 (in equimolar concentrations) suppress NF-kB binding to DNA, around 10 to 30 minutes after stimulation (Fig. 1).

We further find that IL-10, IT9302 and novel analogues also modify the binding of NF-kB to the IL-8 "kB motif" as well as to the p53 "kB motif" oligonucleotides.

| Target genes for NF-kB and their "kB" motifs | | |
|--|------------------------------|-------------------------|
| Human IL-8 promoter | Binding protein | Ref. |
| 'GGAATTTCTC' | Rel A (transcription factor) | Mukaida et al. (1990) |
| Human p53 promoter | NF-kB (p50/p50) | Kirch et al. (1999) |
| 'TGGGGTTTCTC' | NF-kB (p50/p50) | Kirch et al. (1999) |
| Human p53 promoter | Rel A (transcription factor) | Wu, H. and G. L. (1994) |
| 'TGGGGTTTCTC' | Rel A (transcription factor) | Wu, H. and G. L. (1994) |
| Inhibitors of NF-kB binding to DNA | | |
| Class 1 | NB1 | Gilmore et al., 1996 |
| Class 2 | NB2 | Gilmore et al., 1996 |
| Class 3 | NB3 | Gilmore et al., 1996 |
| Class 4 | NB4 | Gilmore et al., 1996 |
| Class 5 | NB5 | Gilmore et al., 1996 |
| Class 6 | NB6 | Gilmore et al., 1996 |
| Class 7 | NB7 | Gilmore et al., 1996 |
| Class 8 | NB8 | Gilmore et al., 1996 |
| Class 9 | NB9 | Gilmore et al., 1996 |
| Class 10 | NB10 | Gilmore et al., 1996 |
| Class 11 | NB11 | Gilmore et al., 1996 |
| Class 12 | NB12 | Gilmore et al., 1996 |
| Class 13 | NB13 | Gilmore et al., 1996 |
| Class 14 | NB14 | Gilmore et al., 1996 |
| Class 15 | NB15 | Gilmore et al., 1996 |
| Class 16 | NB16 | Gilmore et al., 1996 |
| Class 17 | NB17 | Gilmore et al., 1996 |
| Class 18 | NB18 | Gilmore et al., 1996 |
| Class 19 | NB19 | Gilmore et al., 1996 |
| Class 20 | NB20 | Gilmore et al., 1996 |
| Class 21 | NB21 | Gilmore et al., 1996 |
| Class 22 | NB22 | Gilmore et al., 1996 |
| Class 23 | NB23 | Gilmore et al., 1996 |
| Class 24 | NB24 | Gilmore et al., 1996 |
| Class 25 | NB25 | Gilmore et al., 1996 |
| Class 26 | NB26 | Gilmore et al., 1996 |
| Class 27 | NB27 | Gilmore et al., 1996 |
| Class 28 | NB28 | Gilmore et al., 1996 |
| Class 29 | NB29 | Gilmore et al., 1996 |
| Class 30 | NB30 | Gilmore et al., 1996 |
| Class 31 | NB31 | Gilmore et al., 1996 |
| Class 32 | NB32 | Gilmore et al., 1996 |
| Class 33 | NB33 | Gilmore et al., 1996 |
| Class 34 | NB34 | Gilmore et al., 1996 |
| Class 35 | NB35 | Gilmore et al., 1996 |
| Class 36 | NB36 | Gilmore et al., 1996 |
| Class 37 | NB37 | Gilmore et al., 1996 |
| Class 38 | NB38 | Gilmore et al., 1996 |
| Class 39 | NB39 | Gilmore et al., 1996 |
| Class 40 | NB40 | Gilmore et al., 1996 |
| Class 41 | NB41 | Gilmore et al., 1996 |
| Class 42 | NB42 | Gilmore et al., 1996 |
| Class 43 | NB43 | Gilmore et al., 1996 |
| Class 44 | NB44 | Gilmore et al., 1996 |
| Class 45 | NB45 | Gilmore et al., 1996 |
| Class 46 | NB46 | Gilmore et al., 1996 |
| Class 47 | NB47 | Gilmore et al., 1996 |
| Class 48 | NB48 | Gilmore et al., 1996 |
| Class 49 | NB49 | Gilmore et al., 1996 |
| Class 50 | NB50 | Gilmore et al., 1996 |
| Class 51 | NB51 | Gilmore et al., 1996 |
| Class 52 | NB52 | Gilmore et al., 1996 |
| Class 53 | NB53 | Gilmore et al., 1996 |
| Class 54 | NB54 | Gilmore et al., 1996 |
| Class 55 | NB55 | Gilmore et al., 1996 |
| Class 56 | NB56 | Gilmore et al., 1996 |
| Class 57 | NB57 | Gilmore et al., 1996 |
| Class 58 | NB58 | Gilmore et al., 1996 |
| Class 59 | NB59 | Gilmore et al., 1996 |
| Class 60 | NB60 | Gilmore et al., 1996 |
| Class 61 | NB61 | Gilmore et al., 1996 |
| Class 62 | NB62 | Gilmore et al., 1996 |
| Class 63 | NB63 | Gilmore et al., 1996 |
| Class 64 | NB64 | Gilmore et al., 1996 |
| Class 65 | NB65 | Gilmore et al., 1996 |
| Class 66 | NB66 | Gilmore et al., 1996 |
| Class 67 | NB67 | Gilmore et al., 1996 |
| Class 68 | NB68 | Gilmore et al., 1996 |
| Class 69 | NB69 | Gilmore et al., 1996 |
| Class 70 | NB70 | Gilmore et al., 1996 |
| Class 71 | NB71 | Gilmore et al., 1996 |
| Class 72 | NB72 | Gilmore et al., 1996 |
| Class 73 | NB73 | Gilmore et al., 1996 |
| Class 74 | NB74 | Gilmore et al., 1996 |
| Class 75 | NB75 | Gilmore et al., 1996 |
| Class 76 | NB76 | Gilmore et al., 1996 |
| Class 77 | NB77 | Gilmore et al., 1996 |
| Class 78 | NB78 | Gilmore et al., 1996 |
| Class 79 | NB79 | Gilmore et al., 1996 |
| Class 80 | NB80 | Gilmore et al., 1996 |
| Class 81 | NB81 | Gilmore et al., 1996 |
| Class 82 | NB82 | Gilmore et al., 1996 |
| Class 83 | NB83 | Gilmore et al., 1996 |
| Class 84 | NB84 | Gilmore et al., 1996 |
| Class 85 | NB85 | Gilmore et al., 1996 |
| Class 86 | NB86 | Gilmore et al., 1996 |
| Class 87 | NB87 | Gilmore et al., 1996 |
| Class 88 | NB88 | Gilmore et al., 1996 |
| Class 89 | NB89 | Gilmore et al., 1996 |
| Class 90 | NB90 | Gilmore et al., 1996 |
| Class 91 | NB91 | Gilmore et al., 1996 |
| Class 92 | NB92 | Gilmore et al., 1996 |
| Class 93 | NB93 | Gilmore et al., 1996 |
| Class 94 | NB94 | Gilmore et al., 1996 |
| Class 95 | NB95 | Gilmore et al., 1996 |
| Class 96 | NB96 | Gilmore et al., 1996 |
| Class 97 | NB97 | Gilmore et al., 1996 |
| Class 98 | NB98 | Gilmore et al., 1996 |
| Class 99 | NB99 | Gilmore et al., 1996 |
| Class 100 | NB100 | Gilmore et al., 1996 |

METHODS

Human monocytes or U937 monocytic cells were cultured in 6×10^6 cells / 3 ml medium, DMEM added 25 mM Hepes and 5% FCS (Hyclone, Logan, UT). Cells were then either non-stimulated or stimulated for 24 hours with 10 ng/ml of rIL-10 or equimolar amounts of IT9302 or its analogues. The following day cells were stimulated with rIL-10 or analogues once more for 30 minutes before stimulation with 30 ng/ml of IL-1 β for 1 hour.

Peptides were designed and synthesized by professor Arne Holm, Royal Veterinary Academy, Copenhagen. The peptides were dissolved in sterile saline before used. Cells were washed in ice cold PBS and nuclear proteins were isolated as previously described (Johansen et al., 2000). Electrophoretic mobility shift assay (EMSA) for NF-kB binding was performed with 2 μ g of nuclear protein added 2 μ l of 32P-labelled NF-kB probe: IL-8 consensus NF-kB 5'-CAAAATCGGGAAATTCCTC-3' or Promega consensus NF-kB 5'-AGTTGAGGGGACTTCCAGGC-3' were purchased from AH diagnostic, Aarhus, Denmark. Supershift reactions included anti-NF-kB/p50 antibody and anti-NF-kB/p50 antibody (Santa Cruz, CA).

Correspondence to:
Christian Grønhøj Larsen, MD, DMSc
Associate Research Professor
Department of Dermatology D, The Marselisborg Centre
Aarhus University Hospital, DK-8000 Aarhus C, Denmark
chr.gjarsen@net.dk

Phone: +45 23398885 (mobile). Can be reached on this number today.

RESULTS

We compared two synthetic IL-10 analogues A2 and A3, which are nona-peptides containing non-natural amino acids, with IL-10 and IT9302 for their ability to block NF-kB binding to specific "kB motifs" (Fig. 2-5).

In a system of human monocytes, IT9302 and A3 more efficiently suppressed NF-kB binding to the IL-8 "kB motif" than to the p53 "kB motif" (Fig. 2 and 3). Also, in repeated experiments, A3 was more potent than both recombinant IL-10 and the nonapeptide IT9302. In fact A3 suppressed the binding to levels below that of the negative control. In U937 cells, the relative NF-kB binding was also suppressed by IL-10, IT9302 as well as the two new analogues (equivalent to 10 ng/ml of rIL-10) in a dose dependent manner.

A3 and A2 analogues were more efficient than IL-10 and IT9302 with respect to inhibiting the binding to the IL-8 "kB motif" while A3 was most efficient with respect to inhibition of the binding to the p53 "kB motif" in U937 cells.

DISCUSSION & CONCLUSION

IxB- α can specifically block NF-kB/p65 binding to DNA Ghosh S., Baltimore D. (1990) and thereby block binding of p65/p65 homodimers to IL-8 "kB motif". In our hands, effective de novo synthesis of IxB- α appears to be dependent on prolonged (overnight) stimulation with IL-10 or its analogues according to our studies. On the other hand, blocking of NF-kB p50/p65 by p50/p50 may occur within 30 minutes. We therefore stimulated the cells twice and this could be of importance for an effective suppression of NF-kB binding to DNA.

In this study we compare the effect of IL-10 and its synthetic analogues with respect to their modulatory capacity on the binding of NF-kappaB to specific motifs, namely the p53 and IL-8 motifs. We find that:

• IL-10 significantly modulate the binding of NF-kappa to the p53 and the IL-8 motifs.

• The C-terminal domain of IL-10, represented by IT9302 appears to be responsible for this effect.

• Structural modifications of IT9302 may improve the potency of this IL-10-like effect.

• Thus, the new IL-10 analogues, which are structural modifications of IT9302 appears to overcome previous stability problems, which are attributed to IT9302 (data not shown). This enhanced stability could explain the increased potency of the new analogues compared to IL-10 and IT9302.

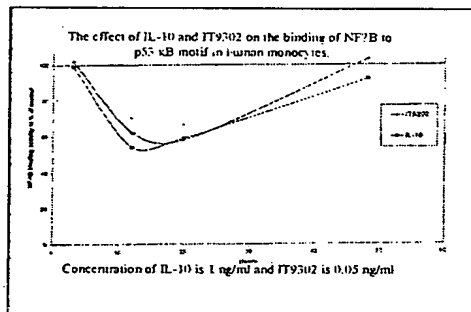


FIG. 1

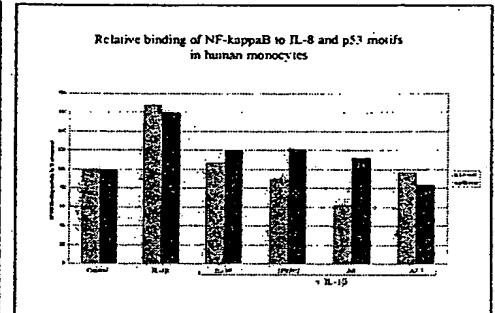


FIG. 2

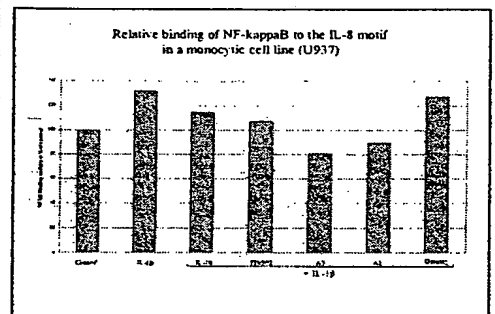


FIG. 3

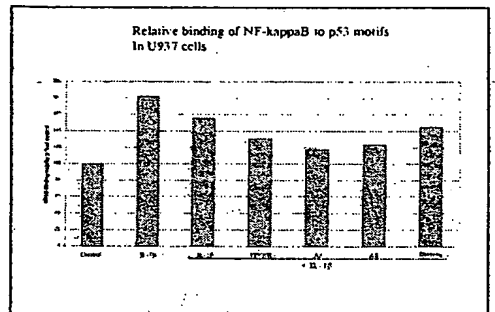


FIG. 4

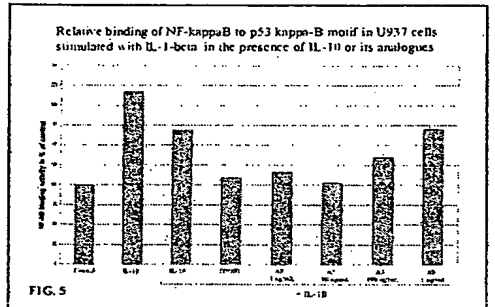


FIG. 5